

AD _____

GRANT NUMBER DAMD17-94-J-4179

TITLE: Her-2/Neu and Breast Cancer

PRINCIPAL INVESTIGATOR: Joni Parker
Doctor Gail Clinton

CONTRACTING ORGANIZATION: Oregon Health Sciences University
Portland, Oregon 97201-3098

REPORT DATE: August 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

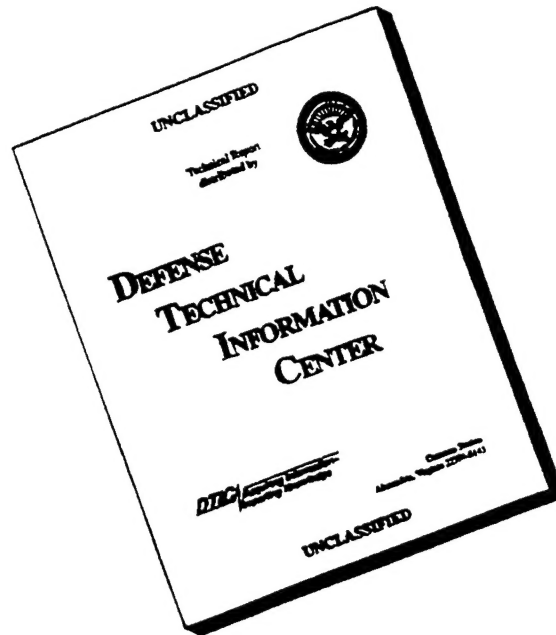
DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19961021 138

DTIC QUALITY INSPECTED 1

DISCLAIMER NOTICE



**THIS DOCUMENT IS BEST
QUALITY AVAILABLE. THE
COPY FURNISHED TO DTIC
CONTAINED A SIGNIFICANT
NUMBER OF PAGES WHICH DO
NOT REPRODUCE LEGIBLY.**

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1996	3. REPORT TYPE AND DATES COVERED Annual (8 Jul 95 - 7 Jul 96)	
4. TITLE AND SUBTITLE Her-2/Neu and Breast Cancer			5. FUNDING NUMBERS DAMD17-94-J-4179	
6. AUTHOR(S) Joni Parker Doctor Gail Clinton				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon Health Sciences University Portland, Oregon 97201-2098			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 2170-25012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) The HER-2/ <i>neu</i> (<i>c-erbB2</i>) protooncogene is overexpressed in up to 30% of breast and ovarian cancers. Overexpression as a 185 kilodalton transmembrane growth-factor-receptor/tyrosine-kinase (p185 ^{HER-2}) is an indicator of poor prognosis. Attempting to characterize the significance of alternative forms of p185 ^{HER-2} , I have focussed on a truncated p95 form comprising the transmembrane and tyrosine kinase domains, and more recently, on an alternative transcript. Specifically, the SKOV-3 carcinoma cell line highly overexpresses HER-2/ <i>neu</i> protein and expresses an aberrant HER-2/ <i>neu</i> transcript of 8 kb, in addition to the "normal" 4.5 kb transcript. This larger transcript may be the product of a gene rearrangement, as SKOV-3 cells exhibit amplification of the HER-2/ <i>neu</i> gene, and Southern blotting experiments indicate a gene rearrangement. Current studies are aimed at characterizing this aberrant HER-2/ <i>neu</i> transcript in terms of quantification, sequence analysis, and identifying its significance in overexpression of HER-2/ <i>neu</i> protein. Since this transcript seems to be selected for in the malignant SKOV-3 cell line, it may confer enhanced oncogenic potential.				
14. SUBJECT TERMS Breast Cancer, HER-2/ <i>neu</i> , <i>c-erbB-2</i> , protooncogene, p185 ^{HER2} , mRNA transcript			15. NUMBER OF PAGES 18	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

JP Where copyrighted material is quoted, permission has been obtained to use such material.

JP Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JP Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

 In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

JP For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

 In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

 In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JP In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jon Parker 8-1-96
PI - Signature Date

TABLE OF CONTENTS

Front Cover	1
SF 298, Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	7
Conclusions	10
References	12
Appendix.....	15

INTRODUCTION:

Nature of the Problem and Background of Previous Work

Characterization of an Alternative HER-2/neu mRNA Transcript

The HER-2/*neu* (*c-erbB-2*) proto-oncogene is developmentally expressed in many tissues. Expression is from a single gene copy which maps to chromosome 17q21. HER-2/*neu* is expressed as a 185 kDa transmembrane tyrosine kinase (p185^{HER-2}) with extensive homology to the Epidermal Growth Factor Receptor (EGFR) (1). The p185^{HER-2} can heterodimerize with EGFR and has been shown to signal through the mitogen-activated protein (MAP) kinase pathway (2), inducing nuclear genetic events that may lead to proliferation and growth of tumor cells. Like EGFR, overexpression of HER-2/*neu* has been reported in an array of human carcinomas (3), most notably in up to 30% of breast and ovarian cancers (3, 4, 5, 6). Moreover, HER-2/*neu* overexpression is associated with constitutive activation, increased tumor invasiveness, malignancy, and poor prognosis for survival of the patient (7).

The mechanism for p185^{HER-2} overexpression and constitutive activation has not been entirely elucidated, although HER-2/*neu* gene amplification, rearrangement, and enhanced transcription have been suggested. Indeed, increased HER-2/*neu* gene copy number has been shown in up to 20% of human breast tumors and many cell lines displaying p185^{HER-2} overexpression (3, 8). However, mechanisms for gene amplification and rearrangement are not well understood, and cannot account for p185^{HER-2} overexpression in up to 10% of human breast cancers that do not exhibit HER-2 gene amplification (3, 6, 8). Therefore, additional mechanisms for overexpression must exist, suggesting tumor cell selection for p185^{HER-2} overexpression in some human breast cancers (3, 5).

As a general rule, cancer cells display genetic instability resulting in mutations, gene amplification, and rearrangement, possibly introducing additional mechanisms for overexpression via altered gene sequence or flanking regions. Examples include: (a) differences in HER-2/*neu* mRNA transcript stability due to gene mutation and/or rearrangement (3, 9); (b) enhanced translation efficiency from altered HER-2/*neu* mRNA transcript due to variances in the 5' and/or 3' untranslated region(s) (9), including gene fusion (e.g., with a highly expressed housekeeping gene, thus leading to HER-2/*neu* expression under control of this gene promoter) or tandem repeats of the HER-2 gene itself; and (c) increased stability of HER-2/*neu* product (e.g., resistance to proteolysis and/or decreased ligand-

binding affinity leading to decreased receptor-ligand-complex internalization or downregulation, possibly coupled with constitutive activation of kinase domain) due to alternative RNA processing which could lead to alterations in intron-exon sequence, such as those seen in the EGFR transcript in human brain tumors (7, 10, 11). Such mechanisms could account for the truncated p95 form of HER-2 identified in our preliminary tissue culture studies (12) and in my preliminary analyses of breast tumor tissues (USAMRDC annual report for July 1994-1995 and unpublished findings).

Moreover, alternative mRNA transcripts for the EGFR, suggested to confer enhanced oncogenicity, have been identified in human brain tumors and cancer cell lines (11, 13, 14). Likewise, alternative HER-2/*neu* mRNA transcripts have, in fact, been identified in both human tumors and tumor cell lines (4, 15, 16). One example of an alternatively spliced HER-2 mRNA has been identified in a gastric carcinoma and encodes a truncated intracellular HER-2 receptor protein (i.e., lacking the kinase domain) (16). Of interest, the SK-OV-3 cancer cell line, which has been shown to overexpress HER-2/*neu* (8), displays an abundant mRNA species of approximately 8kb that strongly hybridizes to a HER-2 cDNA probe (see figure 1 and reference 18), in addition to the well-characterized and cloned 4.5 kb HER-2/*neu* transcript that is normally seen in Northern blotting experiments performed on tumor tissues and cell lines (3, 17, 18). In SK-OV-3, restriction fragment length polymorphism has been demonstrated in the genomic HER-2/*neu* sequence, suggesting gene rearrangement or mutation (4, 8, 15) as a possible mechanism leading to alternative RNA processing. However, the larger mRNA transcript has not yet been characterized.

The fact that this alternative transcript appears to be selected for in this aggressive cancer cell lines suggests that it may be contributing to tumorigenesis. One possibility is that this alternative transcript may facilitate overexpression of HER-2/*neu*, thus contributing to tumorigenesis; however, numerous other possibilities for its selection in these cells exist. In order to determine the function of this alternative transcript, it will be necessary to determine the sequence and characterize its translation product.

The current study is thus aimed at characterizing this alternative 8 kb HER-2/*neu* transcript in SK-OV-3 cells, in terms of identifying sequence alterations that potentially contribute to p185^{HER-2} overexpression, activation, and tumorigenicity. In vitro translation and transfection experiments will be used to characterize its function in these cells. These results will then be used to create novel oligonucleotide primers specific to the alternative sequence that will enable rapid screening of patient breast tumor tissues via RT-PCR for presence of alternative HER-2/*neu* transcript.

Purpose of the Present Work

The purpose of the present work on this project is to elucidate the role of an alternative mRNA transcript for HER-2/*neu* with respect to HER-2/*neu* expression. Such an alternative aberrant transcript could account for upregulation of HER-2 expression and, possibly, an alternative protein product with enhanced oncogenicity.

Methods of Approach

In order to isolate and characterize the aberrant HER-2/*neu* transcript, the following methods are applied: (1) Southern blotting experiments on genomic DNAs to determine the extent of HER-2/*neu* gene amplification and to determine gene rearrangement patterns; (2) Amplification of the divergent HER-2/*neu* mRNA region in the aberrant 8 kb transcript via PCR, subcloning and sequencing; (3) Size-based isolation of the aberrant transcript, subcloning, and sequencing of the alternative mRNA transcript from this overexpressing SKOV-3 cell line; (4) Screening the National Library of Medicine (NCBI) and GenBank databases for sequence similarities with the region of divergence to attempt to either (a) identify its origin within the genome (or homology therein, possibly suggesting a gene rearrangement), or (b) enter the novel sequence into GenBank; (5) Creating aberrant-HER-2-sequence-specific oligonucleotide probes to screen breast tumor tissue samples for expression of an alternative transcript, and/or to use as primers in an RT-PCR-based assay on tumor tissues.

BODY:

Experimental Methods

In order to determine the mechanism of HER-2/*neu* protein overexpression, I have further quantified the level of HER-2 overexpression at the genomic level (i.e., amplification), mRNA level, and protein levels, I have completed Southern blotting on genomic DNA from SKOV3 cells, Northern and Dot blotting of mRNAs, and protein dot blotting of SKOV3 lysates, probing these with specific HER-2 probes.

For Southern blots, 6 μ g of genomic DNA from cells expressing different levels of p185^{HER-2}, including IVAN (epithelial cell line expressing low level p185^{HER-2}), SKOV-3 (highly overexpress p185^{HER-2}), and BT474 (breast carcinoma cell line expressing high levels of p185^{HER-2}) cells were digested with an excess of HindIII, BamHI, or EcoRI and electrophoresed on 0.8% agarose gels. Gels were then transferred to Nytran in 10X SSC (3 M NaCl, 0.3 M Sodium Citrate, according to

standard protocols (6)) and sequentially hybridized with either an $\alpha(32\text{P})\text{dCTP}$ -labelled cDNA probe of 530 bp corresponding to the 5' coding sequence of HER-2/*neu* (base pairs 1400-2000; EcoRI-Sph1 fragment from p9701), or an $\alpha(32\text{P})\text{CTP}$ -labelled 470 bp antisense riboprobe, which is complementary to the 3' HER-2 sequence encoding its extreme C-terminal end.

Protein dot blotting was performed using two-fold serial dilutions of cell lysates with known protein concentration (determined by BioRad protein assay), applied to Nitrocellulose via dot blot manifold with constant suction and probed, in a manner similar to Western blotting, with a-HER-2 antibody 1275, as previously described (12).

For all Northern blotting experiments and other RNA isolations, SK-OV-3 cells were grown to confluency on 15 cm plates, lysed in the presence of proteinase K, and poly A+ RNA (mRNA) extracted over oligo-dT cellulose chromatography columns. For standard Northern blots, 3 mg of mRNA per lane were electrophoresed on 1.2% agarose-formaldehyde gels, which were then transferred to Nytran in 10X SSC, and hybridized, under RNase-free conditions, with either of the HER-2 specific probes, as for Southern blotting experiments described above. RNA dot blots were done on Nytran as well using two-fold serial dilutions of mRNA, beginning with 3 μg for SKOV-3 and 6 μg for IVAN in a total volume of 10 μl . Samples were diluted in DEPC-treated water, heated to 65 C for 15 min. and applied under constant suction to each well of a BRL Life Technologies (Bethesda, MD) 72-well dot blot manifold.

Several approaches were considered for determining the sequence and structure of the aberrant transcript identified in Northern blotting experiments (18). First, RT-PCR was performed on polyA+RNA using reverse transcriptase (MMLV-RT, GIBCO BRL) to form cDNA and then subjecting the reaction to 30 cycles of the PCR (94 C X 30", 65 C X 45", 72 C X 3 min). PCR primers included: an oligo-dT antisense primer, an outer sense primer specific to a region of the HER-2/*neu* gene within the coding region near its 3' end (CAG CCT TCG ACA ACC TCT, Gibco BRL) and an inner, or "nesting" sense primer adding an EcoR1 restriction site (to aid in the subcloning of amplified PCR products for sequencing, i.e. 3' Rapid Amplification of cDNA Ends or RACE) to a slightly more 3' location within the HER-2 coding sequence (GCA CGA ATT CGA GAA CGC AGA GTA CCT G, Gibco BRL). Figure 2 (appendix) schematicizes this methodology. Reactions, including PCR with these primers on (a) full-length HER-2/*neu* expression plasmid (p9002) as a positive control for the HER-2/*neu* cDNA and (b) Bluescript pSK (Stratagene)--without the HER-2/*neu* sequence--as a negative control, were electrophoresed on 0.8% agarose gels, Southern blotted and probed with a $\gamma(32\text{P})\text{-dATP}$ -labelled oligonucleotide (CCT GGG TCT GGA CGT GCC AGT G, Gibco BRL) specific to a region within the HER-2/*neu* sequence to be amplified. The expected size for the positive control amplified product is approximately 600 bp, corresponding to the 3' UTR in the normal 4.5 kb transcript. If the larger size of the alternative transcript could be

accounted for in 3' untranslated sequence, a larger band than 600 bp would be expected to hybridize with the probe in reverse transcribed SK-OV-3 mRNA upon Southern blotting. This PCR product could then be rerun on a DNA-preparatory agarose gel (0.8%), stained with Ethidium Bromide, UV-illuminated, excised according to size indicated by Southern blot hybridization and adjacent (simultaneously electrophoresed) DNA ladder, and eluted for purification, subsequent subcloning (into a pUC8 vector, positive clones will be selected by filter hybridization, with subsequent plasmid DNA purification) and sequencing.

The second method I have attempted, as backup for the RT-PCR reactions to reveal the location of alternative sequence, is the high resolution separation of mRNA transcripts by size on agarose gels, followed by electroelution in dialysis bags and purification (19). Briefly, SK-OV-3 mRNA is currently being used to isolate and sequence the alternative 7.5-8 kb HER-2/*neu* transcript by electrophoresis on denaturing 1.2% agarose gels, isolating bands of the appropriate sizes (according to adjacently run RNA ladder) directly from the gel and electroeluting into 0.5XSB3 (20 mM Tris, 2.5 mM NaOAc, 0.5 mM EDTA, pH 7.9) in RNase-free dialysis bags (Spectrum), then ethanol precipitating mRNA using glycogen as a carrier. Recovered mRNA is then dot blotted (as described for mRNA above) to identify fractions containing HER-2 transcript (see fig. 3A) and Northern blotted to assess integrity of mRNAs (fig. 3B). The intact mRNAs representing 8 kb and 4.5 kb HER-2 transcripts will then be converted into the more stable cDNA form via reverse transcription for subcloning and sequencing.

Results Obtained

Protein expression analysis has revealed ten-fold overexpression of HER-2/*neu* in SKOV-3 cells as compared to noncancerous cell line expression values measured by laser densitometry analysis (fig. 4B) of dot blotted cell lysates (beginning with 150 µg protein for SKOV-3 and 50 µg cellular protein for IVAN (a normal epithelial cell line), in two-fold serial dilutions, fig. 4A). Northern (fig. 1B) and Dot blotted (fig. 5A) cellular mRNAs also reveal at least a ten-fold overexpression at the transcriptional level when analysed by phosphorimaging (fig. 5C) using β-actin as a control for amount of mRNA loaded (fig. 1B, bottom panel, 5A & B). Both HER-2/*neu* transcripts hybridize with both the 5' and 3' HER-2 sequence using corresponding probes, as described in methods, and both give a similar distribution (data not shown), indicating that the full HER-2 sequence is contained in the larger transcript, and that this transcript is more abundant in the cell. Table 1 shows the relative amounts of mRNA expressed as 8 kb and as 4.5 kb transcript as compared to protein overexpression values, indicating that the larger transcript may be translated into wild-type HER-2/*neu* protein, because the relative amount of 4.5 kb transcript overexpression alone cannot account for the level of HER-2/*neu* protein overexpression observed in these cells.

Results of Southern blotting analysis are summarized in Table 2, and generally agree with previously reported findings of evidence for gene rearrangement in SK-OV-3 cells, as indicated in the table references (20, 21, 22, 23, 24, and 25).

For the completed RT-PCR experiments to date, no bands larger than 600bp were seen (see fig. 6, appendix), leaving two possibilities: either (1) the extra sequence in the larger transcript for HER-2 is not contained in the 3' UTR, or (2) the PCR is not efficiently amplifying the larger transcript due to competition from the shorter (and therefore, more readily amplified), "wild-type" transcript. Hence, 5' RACE will be performed by similar methodology, in order to look for differences in the 5' UTR of the alternative transcript. If both 3' and 5' RACE yield no enlightening results, RT-PCR using sense and antisense oligos within the coding sequence will be performed. This would be expected to reveal changes in intron-exon sequences within the coding region of the alternative transcript. For these RT-PCR reactions, however, a newly released, more processive polymerase, such as Expand High Fidelity PCR System (Boehringer Mannheim) or Elongase (GIBCO BRL), will be used for efficient amplification of large products.

CONCLUSIONS:

Implications of Completed Research

We have identified an alternative protein product for the HER-2/*neu* protooncogene that is expressed in human breast cancer samples (Christianson et al., manuscript in preparation). I am now investigating mechanisms for p185^{HER-2} overexpression and focussing on the role of an alternative HER-2/*neu* transcript in a carcinoma tissue culture model.

Results obtained thus far, both in our laboratory and by others who have studied mechanisms of HER-2/*neu* overexpression, indicate that tumor cell lines expressing high levels of HER-2/*neu* tend to exhibit amplification of the HER-2/*neu* gene, and may harbor gene rearrangements that induce overexpression via altered promoter control mechanisms. Overexpression of an alternative HER-2/*neu* mRNA transcript in SK-OV-3 cells may be a product of such rearrangement or translocation events. Additional or aberrant sequence may result in a more stable mRNA (e.g., with alternative secondary structure formations) and possibly an increased rate of translation. It will be important to elucidate the role of this alternative transcript in cell transformation, as it may confer enhanced oncogenicity. Determining the aberrant sequence of this transcript and identifying the protein product it encodes will enable synthesis of specific oligonucleotide probes and/or specific antibodies to detect the presence of this HER-2/*neu* form in human tumors. Such probes will be used to screen patient breast tumor tissues obtained from Ed Keenan's Breast Tumor Repository. These studies will provide the first epidemiological data on the presence of an alternative HER-2/*neu* gene product.

Future Work

I plan to continue the RT-PCR experiments as described in "experimental methods" and "results obtained" sections above, as well as electroeluting mRNAs from agarose gels for separation by size, subsequent reverse transcription, subcloning, and sequencing.

Novel sequence obtained through the RT-PCR and subcloning experiments described will be entered in the database GenBank and compared for sequence homology with any currently known segments of DNA or RNA that may elucidate the functional significance of the alternative transcript sequence.

Once the unique sequence of the alternative transcript is determined, specific oligonucleotide probes will be generated in order to clone the cDNA of the alternative transcript via reverse transcription. This cDNA sequence will then be used to assess the functional significance of the alternative transcript in cell growth and transformation via transfection experiments. In vitro translation experiments with purified alternative mRNA transcript will also be performed in order to characterize the protein product. In addition, transcription initiation rate and mRNA stability of the alternative transcript will be assessed.

To assess statistical significance, many breast tumor tissues will need to be analyzed via RT-PCR with specific oligonucleotide primers that will amplify the aberrant region within the HER-2/*neu* transcript. Appropriate controls must also be used to rule out artifact. Such controls will necessarily include normal breast epithelium obtained from breast reduction mammoplasty and immunoprecipitation techniques. Samples will also need to be analyzed further for: (1) alternative translation products corresponding to the alternative HER-2/*neu* transcript; (2) correlation between overexpression at the protein level of HER-2/*neu* with presence of (a) gene amplification and/or re-arrangement, (b) overexpression at the transcription level, and (c) presence of an alternative transcript; and (3) follow-up data obtained regarding clinical outcome.

References

1. Coussens, L., Yang-Feng, T.L., Liao, Y.-C., Chen, E., Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230: 1132-9, 1985.
2. Ben-Levy, R., Paterson, H.F., Marshall, C.J., and Yarden, Y. A single autophosphorylation site confers oncogenicity to the neu/erbB-2 receptor and enables coupling to the MAP kinase pathway. *The EMBO Journal* 13(14): 3302-11, 1994.
3. Hynes, N. E. and Stern, D. F. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochimica et Biophysica Acta* 1198: 165-84, 1994.
4. Hung, M.-C., Zhang, X., Yan, D.H., Zhang, H.-Z., He, G.-p., Zhang, T.-q., and Shi, D.-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Letters* 61: 95-103, 1992.
5. Lonn, U., Lonn, S., Nilsson, B., and Stenkvist, B. Intratumoral heterogeneity for amplified genes in human breast carcinoma. *Internal Journal of Cancer* 58: 40-5, 1994.
6. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., and Press, M.F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 24: 707-12, 1989.
7. Collins, V.P. Epidermal growth factor gene and its transcripts in glioblastomas. *Recent Results in Cancer Research* 135: 17-24, 1994.
8. Tyson, F.L., Boyer, C.M., Kaufman, R., O'Briant, K., Cram, G., Crews, J.R., Soper, J.T., Daly, L., Fowler, W.C., Jr., Haskill, J.S., and Bast, R.C., Jr. Expression and amplification of the HER-2/neu (c-erbB-2) protooncogene in epithelial ovarian tumors and cell lines. *American Journal of Obstetrics & Gynecology* 165(3): 640-6.
9. Tanguay, R.L. and Gallie, D.R. Translational efficiency is regulated by the length of the 3' untranslated region. *Molecular and Cellular Biology* 16(1): 146-56, 1996.
10. Callaghan, T., Antczak, M., Flickinger, T., Raines, M., Myers, M., and Kung, H.-J. A complete description of the EGF-receptor exon structure: implication in oncogenic activation and domain evolution. *Oncogene* 8: 2939-48, 1993.
11. Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S., and Vogelstein, B. Structural alterations of the epidermal growth factor

receptor gene in human gliomas. *Proceedings of the National Academy of Sciences, USA* 89: 2965-69, 1992.

12. Lin, Y.H. & Clinton, G.M. A soluble protein related to the HER-2 protooncogene product is released from human breast carcinoma cells. *Oncogene*, 6: 639-43, 1991.

13. Ekstrand, A.J., Sugawa, N., James, C.D., and Collins, V.P. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proceedings of the National Academy of Sciences, USA* 89: 4309-13, 1992.

14. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P.H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309: 418-25, 1984.

15. Fujimoto, J., Hori, M., Ichigo, S., Misao, R., and Tamaya, T. Preliminary study of oncogene expressions in endometrial cancers: Aberrant estrogen receptor gene and erbB-2 expressions. *European Journal of Gynaecological Oncology* 16(1): 40-7, 1995.

16. Scott, G.K, Robles, R., Park, J.W., Montgomery, P.A., Daniel, J., Holmes, W.E., Lee, J., Keller, G.A., Li, W.-L., Fendly, B.M., Wood, W.I., Shepard, M., and Benz, C.C. A truncated intracellular HER2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. *Molecular and Cellular Biology* 13(4): 2247-57, 1993.

17. Karlan, B.Y., Jones, J., Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecologic Oncology* 53: 70-77, 1994.

18. Lichtenstein, A., Berenson, J., Gera, J.F., Waldburger, K., Martinez-Maza, O., and Berek, J.S. Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: Correlation with expression of HER-2/neu oncogenes. *Cancer Research* 50: 7364-70, 1990.

19. Harty, R.N. & O'Callaghan, D.J. Isolation and purification of specific, overlapping mRNA species by electroelution from agarose gels. *Biotechniques* 10(6): 728-9, 1991.

20. Jones, J., Lagasse, L.D., and Karlan, B.Y. Steroid hormone independence of HER-2/neu mRNA expression in four human ovarian cancer cell lines. *Gynecologic Oncology* 55: 421-6, 1994.

21. Karlan, B.Y., Jones, J., Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecologic Oncology* 53: 70-7, 1994.
22. Zhang, X., Silva, E., Gershenson, D., and Hung, M. Amplification and rearrangement of c-erbB proto-oncogenes in cancer of human genital tract. *Oncogene* 4: 985-9, 1989.
23. Kraus, M.H., Popescu, N.C., Amsbaugh, S.C., and King, C.R. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *The EMBO Journal* 6(3): 605-10, 1987.
24. Tyson, F.L., Boyer, C.M., Kaufman, R., O'Briant, K., Cram, G., Crews, J.R., Soper, J.T., Daly, L., Fowler, W.C., Haskill, J.S., and Bast, R.C, Jr. Expression and amplification of the HER-2/neu (c-erbB-2) protooncogene in epithelial ovarian tumors and cell lines. *American Journal of Obstetrical Gynecology* 165(3): 640-6, 1991.
25. Hung, M-C., Zhang, X., Yan, D-H., Zhang, H-Z., He, G-p., Zhang, T-q., and Shi, D-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Letters* 61: 95-103, 1992.

Appendix

APPENDIX:

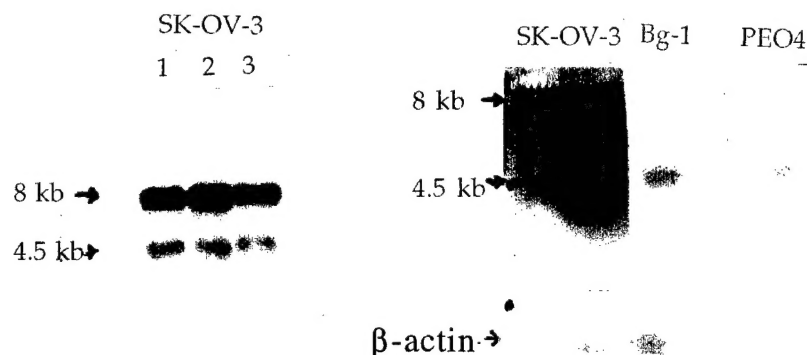


Figure 1. Northern blots of poly-A selected mRNA from SK-OV-3 cells (A, lanes 1-3, 1.5 mg per lane) and SK-OV-3, Bg-1, Bg-1 supplemented with estradiol (E2), and PEO4 carcinoma cell lines (B, lanes 1, 2, 3, and 4, respectively, 3 mg per lane) probed with a(32P)dCTP random prime labelled 5' HER-2 cDNA sequence (A and top panel of B) and B-actin as a control for the amount of mRNA loaded in each lane (B, bottom panel).

3751 GGA GGA GCT GCC CCT CAG CCC CAC CCT CCT CCT GCC TTC AGC CCA GCC TTC GAC
GCAC+\\\\\\OUTER OLIGO

PRIMER\\\\

AAC CTC TAT TAC TGG GAC CAG GAC CCA CCA GAG CGG GGG GCT CCA CCC AGC ACC

TTC AAA GGG ACA CCT ACG GCA GAG AAC GCA GAG TAC CTG GGT CTG GAC GTG CCA
GCACGAATT+\\\\\\INNER OLIGO PRIMER/////

GTG TGA 3918.....(3' UTR).....4530 AAAAAAAAAAAAAAAAAA

Figure 2. Extreme 3' coding sequence beginning at nucleotide residue #3751 and ending with a stop codon (TGA) at #3918. For simplicity the 612 bp sequence of the published (ref. #1) 3' untranslated region is not shown, but appears schematically, ending with the poly-A tail. 5'-3' PCR primers are indicated as "outer" and "inner" (single underlined sequence), and the sequence of the oligonucleotide used as a probe (double underlined sequence, overlaps with inner oligo by four base pairs at 3' end) is indicated, as described in the text.



Figure 3. Electroeluted mRNA hybridizes with the 5'HER-2 cDNA sequence. (A) Dot blot of electroeluted mRNAs from 5 mm gel slices numbered 1 (top of gel) through 6 (towards bottom of gel) corresponding to sizes of approximately 8.5-10 kb (1), 7.5-8.5 kb (2), 6.5-7.5 kb (3), 5.5-6.5 kb (4), 4-5.5 kb (5), and 3-4 kb (6); and (B) Northern blot of electroeluted mRNA from fractions 2, 4, 5, and 6 in (A) above (lanes 2, 3, 4, and 5, respectively) electrophoresed alongside SKOV-3 mRNA (lane 1). Both blots, in (A) and (B) above, were hybridized with an α (32P)dCTP-labelled 9701 HER-2 cDNA probe.

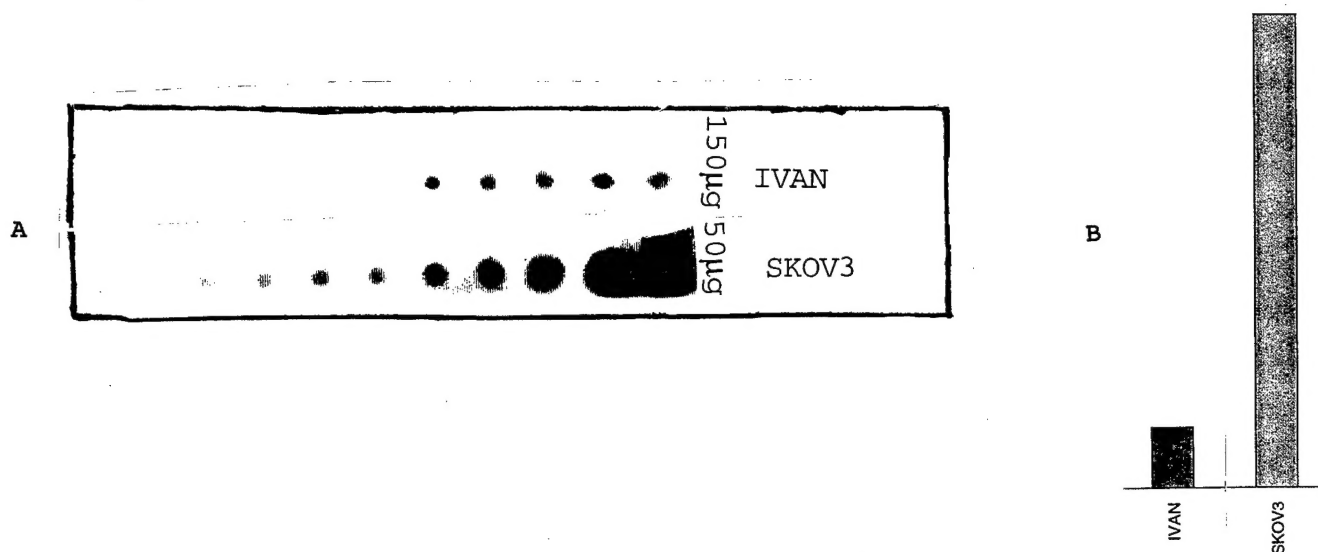


Figure 4. Relative protein overexpression of p185^{HER-2} in SKOV-3 cells vs. IVAN epithelial cells. (A) Total cell lysate was applied to Nitrocellulose via dot blot manifold in two-fold serial dilutions from 150 µg for IVAN cells and from 50 µg for SKOV-3 cells. Blots were blocked in 10% nonfat milk and probed with α-HER-2 antibody 1275, according to standard protocol as previously described (12). (B) Autoradiograph analysis by laser densitometry provided values expressed as relative p185^{HER-2} protein expression levels for IVAN vs. SKOV-3, as indicated.

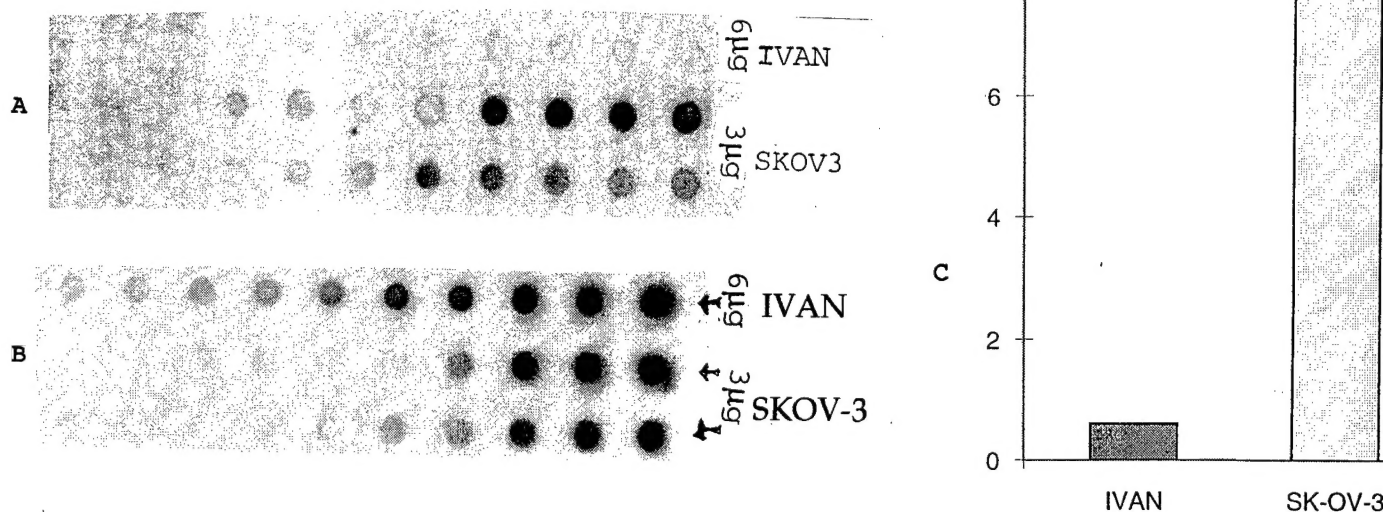


Figure 5. Relative HER-2/neu mRNA expression levels in IVAN and SKOV-3 cells. (A) Phosphorimaging of dot-blotted poly-A⁺-selected mRNAs from IVAN and SKOV-3 cells, as indicated, in two-fold serial dilutions from 6 µg for IVAN and from 3 µg for SKOV-3, probed with α(32P)dCTP-labelled 5'HER-2 cDNA probe (1.8 kb XhoI-SphI fragment from p9701), as described in Methods. (B) Phosphorimage of dot blot in (A), stripped and reprobed with α(32P)dCTP-labelled B-actin cDNA probe. (C) Relative levels of total HER-2 mRNA (i.e., regardless of size of transcript) expression in IVAN vs. SKOV-3 cells as determined by phosphorimager analysis of dot blots, controlling for amount of mRNA loaded with B-actin levels, and averaging, over 12 serial dilutions, the values for fold-overexpression obtained by direct comparison of IVAN vs. SKOV-3 for each dilution.

	IVAN	SK-OV-3
total HER-2 protein	1	10
total HER-2 mRNA	1	<div> <div>10</div> <div> <div>4.5kb</div> <div>30%</div> </div> <div> <div>8 kb</div> <div>70%</div> </div> </div>

Table 1. Comparison of HER-2 mRNA to p185^{HER-2} protein fold-overexpression in SKOV-3 carcinoma cells as compared to IVAN epithelial cell lines, obtained from data-analysis shown in figures 4(B) and 5(C). Data averaged from several phosphorimager analyses of Northern blots of SKOV-3 mRNA, probed with both 5'HER-2 cDNA (as in figure 1) and 3'HER-2 antisense riboprobe (data not shown) was used to obtain the relative amounts, expressed in percentages, of 8 kb and 4.5 kb transcripts.

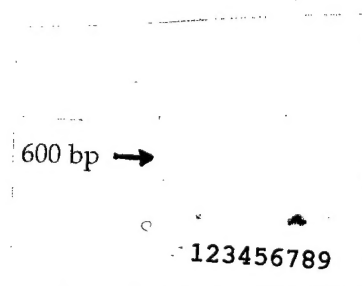


Figure 6. Southern blot of RT-PCR products electrophoresed on 0.8% agarose and probed with (32P)dCTP end-labelled HER-2 probe, as described in text, showing hybridization with no products larger than about 600 bp.

Genomic HER-2/neu: Restriction Fragment Length Polymorphisms

c-erbB-2 specific probe used	Restriction Fragment Lengths				
	EcoR1	BamH1	HindIII	Pst1	Xba
890 bp 3' coding = EcoR1 fragment of HER-2 cDNA (Jones et al., 1994 (20); Karlan et al., 1994 (21))	12.5 kb "SKOV-3 only": 5.8 kb		14.5 kb		
3.0 kb extreme 3' coding & 3' UTR = Kpn(3222)- HindIII fragment from pCER204 (Zhang et al., 1989, ref. #22)	6.6 kb 5.8 kb 4.4 kb (9.6 kb, 2.1 kb)				
800 bp 3' coding region from Acc1 fragment (2200 to 3000bp) of HER-2 cloned into pMAC30 (Kraus et al., 1987 (23); Tyson et al., 1991 (24))	6.6 kb 4.4 kb (3.8 kb, 3.4 kb)				
"full-length" c-erbB-2 from pSV2erbB-2 (Hung et al., 1992, ref. # 25)	12.5 kb (5.8 kb)	(23 kb) 12.5 kb 7 kb 6 kb 1.8 kb	12.5kb (12.5kb) 4 kb	12.5kb 6 kb 3.6kb 2.8kb 1.7kb 1.1 kb 0.9kb	9.4kb 6.6kb 5.8kb (7.6, 4kb) 2.5kb
530 bp 5' EcoR1-Sph1 fragment (1400-2000bp) from p9701--5' coding (for ECD) HER-2 cDNA (Joni Parker, unpublished findings)	12.5 kb (5.8 kb)	7 kb 6 kb	12.5 kb 1.5 kb		
470 bp riboprobe--3' coding (for KD) from pSP6400 (Joni Parker, unpublished findings)	12.5 kb 4.4 kb	23 kb 12.5kb	12.5 kb		

Table 2. Summary of restriction length polymorphisms obtained from Southern blotting of SKOV-3 genomic DNA, as compared to IVAN cell DNA. Results from several groups are shown, varying with the HER-2-specific cDNA probe used. Aberrant restriction fragment lengths only observed in SKOV-3 to hybridize with HER-2 probes, as indicated, are in parentheses.